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CATIONIC & AMPHOTERIC SURFACTANT PRIMARY BIODEGRADABILITY RING TEST

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CONTENTS

LIST OF TABLES

SUMMARY

The use of surfactants in detergents within the EU is bound by a number of Directives 73/404/EEC (EC 1973a), 73/405/EEC (EC 1973b), 82/242/EEC (EC 1982a) and 82/243/EEC (EC 1982b) and 86/94/EEC which bring together the laws of the Member States relating to methods for biodegradability testing. These Directives ensure that only those surfactants which are adequately biodegradable are placed on the Common Market. The present system ensures that all anionic and non-ionic surfactants present in detergents must be tested for primary biodegradability. Although the original Directive (73/404/EEC) referred also to cationic and amphoteric surfactant groups, standardised primary biodegradability test methods are not as yet approved.

A ring test has been performed to assess the validity of the standardised test methods for cationic and amphoteric surfactants in the draft proposal for a Directive, currently undergoing an adoption procedure by the Commission. Six laboratories volunteered, one each from the UK, Netherlands, France, Portugal, Germany and Italy volunteered, to carry out the tests on six test compounds: a hard and soft anionic surfactant for reference purposes, two cationic surfactants (a dimethyl-dialkyl ammonium salt, and a diesterquat) and two amphoteric surfactants (a coco-amido betaine and a dimethylamine betaine).

Primary degradation experiments were carried out using inocula taken from local sewage treatment works, final effluent. Analytical detection was performed using colorimetric methods, methylene blue for the anionic reference surfactants, disulphine blue for the cationics and orange II for the amphoterics. One of the objectives was an assessment of the analytical methodologies used for the cationic and amphoteric surfactants, given that they are not generally used on a routine basis.

Although the analytical techniques appeared to work satisfactorily, some laboratories reported operational difficulties regarding manipulation of the solvent solutions. It was therefore recommended that the methodologies be carefully documented and offered as guidelines accompanying the Directive. It was also recommended that alternatives to chloroform should be considered as the extraction solvent for both methods, due to its toxic nature. In addition, owing to the wide spectrum of amphoteric and cationic surfactants available, it was recommended that further tests should be undertaken to assess the applicability of the disulphine blue and orange II methods to other classes of cationic and amphoteric surfactants respectively.

Overall, more than 90% of the standard soft anionic surfactant was removed after 19 days which demonstrated that the inocula were sufficiently active (Table 1.1). One inoculum was less active giving only 70% removal, but this inoculum was able to biologically remove high proportions (95 –100%) of the three other surfactants tested. Results for the hard anionic surfactant standard were much more variable. Four laboratories reported less than 15% removal, but the other two each gave 70%. The pattern of results reported for the cationic and amphoteric surfactants by the latter two laboratories suggested that the inocula were not ‘over active’ but potentially better acclimated to the hard standard. Neither standard was degraded or removed abiotically.

Amphoteric B was adequately biodegraded and amphoteric A was probably also biodegraded. The evidence for the biodegradability of cationic B was less convincing, since

two values of around 70% were obtained from the abiotic test. There is conflicting evidence concerning cationic A, largely owing to operational difficulties attributed to the strong sorption potential of cationic surfactants. As a consequence its biodegradability status is unknown. Further investigations into the two cationics are required.

Table 1.1 Summary of results of 2nd EU ring test

Surfactant	Mean % removal	Standard deviation (%)	n (laboratories)
Soft Anionic	94	12	6
Hard Anionic	27	34	6
Cationic A	63	35	4
Cationic B	92	10	6
Amphoteric A	99	2	6
Amphoteric B	97	4	5

Table 1.2 Results from the individual laboratories

	Soft Anionic		Hard Anionic	
Lab No.	**% Removal - Total**	**Abiotic**	**% Removal - Total**	**Abiotic**
1	98	-	0	-
2	100	-	13	-
3	99	0*	70	0*
4	99	-	70	-
5	96	0	1	1
6	70	2*	9	5*
	Cationic A		**Cationic B**	
Lab No.	**% Removal - Total**	**Abiotic**	**% Removal - Total**	**Abiotic**
1	35	-	99	-
2	89	-	96	-
3	98	94*	95	78*
4	28	70*	95	0*
5	-	-	71	70
6	-	-	95	1*
	Amphoteric A		**Amphoteric B**	
Lab No.	**% Removal – Total**	**Abiotic**	**% Removal – Total**	**Abiotic**
1	99	-	99	-
2	96	-	97	-
3	100	25*	89	0*
4	100	0*	98	3*
5	98	98	-	-
6	100	1*	100	1*

* Inhibited with mercuric chloride

1. OBJECTIVES

The overall objective of the study was to validate previously standardised test methods to be used in the updated Community detergent Directives 73/404/EEC, 73/405/EEC, 82/242/EEC and 82/243/EEC for cationic and amphoteric surfactants.

The specific objectives of the study were as follows:

a) to gain the participation of at least 6 laboratories in the validation exercise;

b) to agree the programme of work to be carried out by the laboratories, including draft guidelines, the type of reference surfactants and the number of repeat tests;

c) to supply the test compounds and then collate and analyse the data generated following the ring-testing exercise;

d) to assess the performance and applicability of the analytical approach.

2. BACKGROUND

The use of surfactants in detergents within the EU is bound by a number of Directives 73/404/EEC (EC 1973a), 73/405/EEC (EC 1973b), 82/242/EEC (EC 1982a) and 82/243/EEC (EC 1982b) and 86/94/EEC which bring together the laws of the Member States relating to methods for biodegradability testing. These Directives ensure that only those surfactants that are adequately biodegradable, are placed on the Common Market. The present scheme ensures that all anionic and non-ionic surfactants present in detergents must be tested for primary biodegradability only, by methods laid down in the Directives.

In the draft proposal for a new Directive, currently undergoing an adoption procedure by the Commission, it is intended that all types of surfactants used in detergents should be tested; cationics and amphoteric surfactants should be included for the first time. They should all be tested for ultimate biodegradability (mineralisation) instead of primary biodegradability, but under some circumstances primary biodegradation should also be measured. Also the testing will be done before marketing so that extraction of surfactants from detergents will not be necessary before testing. Not only will this remove a costly and tedious process but it will also result in the knowledge that a specific surfactant, rather than a class of surfactants, is being examined.

The existing ISO Standard 14593 (1999; headspace CO_2 evolution in sealed vessels) was chosen for application to the measurement of ultimate biodegradation of all types of surfactant and has recently been subjected to an international ring test (EU 97-501089; WRc May 1999).

The official OECD scheme (OECD 1976) for assessing primary biodegradability, which has been carried over into the measurement of ultimate biodegradability (OECD, 1981) consists of two parts:

a) a screening, batch shake-flask die away test with acceptance of products reaching the required value of biodegradation;

b) and a dynamic simulation test (confirmatory) for any product not satisfying the screening test requirements: only the confirmatory test results are to be taken as the basis for decisions in dispute.

This scheme is based on the findings that surfactants which degraded to the acceptable level in the screening test were also removed to at least the same extent in the confirmatory test; some which passed the latter did not pass the former.

The existing Directives describe, in annexes, semi-specific colorimetric tests and the confirmatory simulation test, but they do not describe the screening die-away test. In the proposed Directive the important, but not sole, criterion for acceptance is to be ultimate biodegradability. Surfactants that fail however, may be tested for primary biodegradability, but must also then be subjected to a special risk assessment.

The present Directives describe the semi-specific colorimetric methods that determine the parent surfactant, but only of anionic and non-ionic surfactants. It has been found that most (>98%) of the synthetic anionic on the market react in the appropriate semi-specific

colorimetric test (Europlus 1997), while soaps do not, and for non-ionics the value is less clear at 60-80%, the alkyl polyglucosides being the largest group which do not respond.

Ultimately instrumental methods (e.g. GC or LC-MS) may be approved for surfactants used in detergents, but which do not react in semi-specific analytical tests for the relevant class of surfactant. The existence of such surfactants does not interfere with ring tests of the assessment of primary biodegradability of cationic and amphoteric surfactants, providing that the current commercially important ones react positively in the semi-specific analytical test.

It was therefore decided to extend the assessment of primary biodegradability in the OECD die-away test to cationic and amphoteric surfactants using colorimetric methods, namely the Disulphine Blue Active Substances (DBAS) and the Orange II respectively.

This study is designed to assess the inter-laboratory performance of the primary biodegradability test guidelines. WRc would co-ordinate the ring-test, collate and analyse the test results and issue a report.

Initial approaches to industry to ascertain if they were willing to support the new study were favourable and the scope of work, timetable and remuneration was agreed with the EC. The initial meeting with the AISE/CESIO Biodegradability Task Force took place, in Brussels, on 10 February 1999. At the meeting the terms of reference, and the proposed ring-test methodology were presented by representatives of WRc. The industry felt that the ring test required further validation work prior to it gaining their support. Such an exercise was, however beyond the scope to this contract so it was agreed with industry that although their laboratories would not be encouraged to take part, they would provide chemicals, and where possible, offer advice with any methodologies. WRc did however, recognise, that some of the reservations expressed by industry were justified and agreed with the EC to perform a limited validation exercise to address the more pertinent points, namely:

- investigate the analytical procedures;
- their applicability to the die-away test, especially in the presence of silica;
- the range of sub-types of surfactants reacting with the reagents.

The voluntary support of 6 laboratories was gained to participate in the ring test and suitable chemicals were either donated by industry or purchased.

3. PLAN

It was intended that the primary biodegradability of two of each type of surfactant would be assessed using the OECD Die Away test, using the extraction and analytical procedures for the determination of cationic and amphoteric surfactants, described in Section 8. One easily degradable (soft) anionic surfactant and one less readily degradable (hard) anionic surfactant were also to be assessed, as reference compounds.

A minimum of six laboratories was sought in order to obtain sufficient data for a satisfactory statistical analysis of the results. The laboratories were asked, as a minimum, to set up triplicates to be analysed at day 0 and after incubation for 19 days. For the two reference surfactants, results obtained with at least a period of incubation for 14 days were to be reported. Any other results, e.g. for shorter periods of incubation than 19 days, could be obtained and would be reported. As a safeguard, participants were asked to check the stability of control solutions (containing about 5 mg l^{-1} of surfactant) in the medium, without the addition of an inoculum, over a period of 19 days.

3.1 Outline of Events

Of the 15 laboratories originally contacted, only 6 agreed to take part, with most industrial laboratories declining the offer due to reasons stated above (see Section 2). The various documents and samples of the six surfactants (kindly supplied by the Industry) were distributed in mid-October 1999. The results were to be returned to WRc by the end of November but in the event three sets of results were received during December, a further two sets during January 2000 and the sixth contribution was received at the end of March. Reasons for the delay were attributed to:

- late receipt and distribution of the surfactants by the co-ordinating laboratory;
- dislocation of effort due to re-location of a participant;
- an unexpectedly heavy work-load at the time of the ring test for one participant.

4. PARTICIPATING LABORATORIES

The laboratories that agreed to take part in the ring test were:

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E-mail: BarnesS@ukorg.huntingdon.com

5. TEST CHEMICALS

Details on the 6 chemicals used for the tests were as follows:

1) Soft Anionic

Di-iso-octylsulphosuccinate-sodium salt
(Di (2-ethyl-hexyl)sulphosuccinate - sodium salt)
Purity 98% M = 444g 53% C (calculated).

2) Hard Anionic

Branched dodecyl benzene sulphonate
Formula: $C12H25.C_6H_4.SO_3H$ (free acid)
Purity 96% M = 326g 68% C (calculated).

3) Cationic (A)

Di(hydrogenated tallow) dimethylammonium chloride
General formula: $[R\text{-}N(CH_3)_2\text{-}R]^+Cl^-$ R = straight alkyl chain (mainly C_{16}-C_{18})
Purity 75% M = 573.5g 40% C (calculated)
Silica required at 5 mg l^{-1} active ingredient = 1 g l^{-1}.

4) Cationic (B)

Diesterquat: (based on partially hydrogenated tallow fatty acids)
CAS No. 93334-15-7 Fatty acids, tallow, reaction products with triethanolamine, di-methyl sulfate-quaternized.

other possible name:
CAS No. 91995-81-2 Fatty acids, C10-20 and C16-18-unsatd., reaction products with triethanolamine, di-methyl sulfate-quaternized.Methyl bis(ethyltallowate)2-hydroxyethyl ammonium methyl sulphate

Purity 90% M= 782 g (approx.) 66% C (calculated, approx.)

5) Amphoteric (A)

(C12-C14)-dimethylamine betaine
Purity 30% M = 293g (calculated) 60% C (calculated)

6) Amphoteric (B)

1-propanaminium 3-amino-N-carboxymethyl N-coco acyl derivatives
Purity 30% M = 354g (calculated) 70% C (calculated)

We would like to thank the chemical industries for their donation of the test compounds.

6. CHEMICAL ANALYSIS VALIDATION EXERCISE

6.1 Introduction

It has been recognised by industry, amongst others, that the "standard" methods described in the Directive do not address certain critical points regarding the determination of amphoteric and cationic surfactants using semi-specific colorimetric analytical techniques.

It was therefore agreed with the EC that we would carry out a limited validation exercise to address the most pertinent points. The issues raised by industry were:

1. applicability to all types of surfactants within each class;

2. analysis of cationic surfactants in the presence of silica (used to alleviate potential toxicity);

3. potential interferences from non-target compounds.

Given that the biodegradation tests were going to be performed on single products, potential interferences from other types of surfactants were not an issue within the terms of reference of this study, and so were not considered further.

6.1.1 Applicability of analytical methods

The EU report (Europlus 1997) lists the diester quaternary cationic surfactants as constituting 99% of the total tonnage of cationics and as being unresponsive to the DBAS method. But French workers report that at least one type of "esterquat" does react when conditions of the test are changed. Neither is the position with amphoterics completely clear; although it is known that alkyl (C_{12}-C_{18}) amido betaines and alkyl (C_8-C_{18}) betaines which contribute to *ca.* 90% of the market, respond in the Orange II test (Boiteux,1984). Betaines of minor commercial importance (e.g. alkyl amido hydroxy-sulpho betaine, and glycinates) would require further investigation.

The following questions were therefore addressed:

1. Does the DBAS method work for esterquat cationic surfactants?

2. Does the Orange II test work for the amphoteric surfactants to be analysed (e.g. amidobetaines, alkyl amido betaines, and sulpho hydroxy betaines)?

6.1.2 Analysis of cationic surfactants in the presence of silica

It has been reported that even at relatively low concentrations (e.g. 5-20 mg l^{-1}), certain cationic surfactants can be toxic to micro-organisms. To overcome this problem, biodegradation tests can be carried out by either adding silica to the test vessel to adsorb the surfactant or by adding the test substance in specific aliquots over a period of time, (as in the AFNOR method). Testing the cationic surfactants is further complicated by the fact that they

adsorb strongly to glass surfaces. This would result in, or lead to serious errors associated with the analysis step if aliquots of sample were simply withdrawn from the vessel. One way of alleviating this problem would be to add silica to the test vessels and then at the appropriate time intervals carry out the colorimetric test within the test vessel. The complexation/extraction procedure would thus recover all of the test substance from the glass and silica.

The question to be answered therefore was:

1. Can the cationic surfactants be quantitatively extracted from a sample containing silica?

6.2 Methods

To assess the applicability of the methodology to be used for the determination of cationic and amphoteric surfactants, the following methods were utilised:

Cationic Surfactants (DBAS) - Disulphine blue is an anionic dyestuff that forms a chloroform-soluble complex with a cationic surfactant, which is then, measured spectrophotometrically (HMSO 'blue book' method - Analysis of surfactants in waters, wastewaters and sludges (1980)). Given that potential interfering substances were excluded from the biodegradation tests, the anion-exchange separation step that allows the determination of cationics in the presence of other anionic components was not required.

Amphoteric Surfactants- The Orange II procedure is also based on the formation of a chloroform-soluble coloured complex of the azo dye and amphoteric surfactant. The complex is again measured spectrophotometrically (Boiteux 1984).

6.3 Laboratory method evaluation

6.3.1 Applicability to cationic surfactants

To test the applicability of the method to a variety of cationic surfactants, an example from three distinct types were tested:

- Cetyl trimethyl ammonium bromide (CTAB);
- Dioctadecyldimethyl ammonium chloride (DODMAC);
- Diesterquat (TEA diester).

Standards solutions of CTAB were prepared at concentrations of 0, 0.1, 0,5 1, 5 mg l^{-1} by dilution of a stock standard with deionised water. A 40 ml sample was placed in a screw top glass tube (60 ml) and to it, added: 5 ml of sodium acetate buffer (115 g anhydrous sodium acetate + 35 ml glacial acetic acid in 1l DIW), 2 ml of disulphine blue solution (0.16 g disulphine blue - CI 42045, (Patent Blue Violet, 70%, Sigma Ltd) dissolved in 18 ml DIW, 2 ml ethanol and diluted into 250 ml of DIW) and 15 ml of chloroform. The tube was shaken vigorously for 90 s and left to separate for 5 minutes.

Immediately after the two phases have separated a Pasteur pipette was used to withdraw carefully the solvent phase and place it in a 1 cm cuvette for determination against a chloroform blank at 628 nm. The blue complex was shown to adsorb strongly to the surface of glass, and so the Pasteur pipette had to be conditioned by withdrawing and discarding the sample three times before use. Additionally, the cuvette was rinsed with methanol between each sample.

Once the calibration had been performed, 1 mg l^{-1} solutions of the other cationic surfactants were prepared and the analysis repeated. Results are presented in Table 6.1.

Table 6.1 Relative response of DBAS method to different cationic surfactants

Substance	Response (Abs/ppm)	Response (Abs/mmole)
CTAB	0.315	0.116
DODMAC	0.191	0.112
TEA diester	0.120	0.094

Results show that for CTAB and DODMAC similar recoveries were obtained when calculated on a molar basis. The sensitivity for the TEA diester was slightly less compared with the other cationics, but still sufficiently high to permit the method to be used for the biodegradation test. Varying the pH by adding different concentrations of buffer (from 0 to 5 ml) did not improve the extraction efficiency.

6.3.2 Applicability to amphoteric surfactants

The Orange II method was used as described by Boiteux (1984), but scaled down from using 100 ml of sample to 40 ml. To each sample, 0.8 ml of 18N sulphuric acid was added followed by 0.8 ml of the Orange II reagent (1 g of Orange II - Sigma Ltd, 95% dye content in 1 litre of DIW containing 0.1M sodium chloride), and finally 7.5 ml of chloroform. The vessel was shaken vigorously for 2 minutes and then allowed to stand until the phases had completely separated (approximately 5 minutes). For higher concentrations of amphoteric surfactant, the sample tended to emulsify and centrifugation was required in order to separate the phases. A Pasteur pipette was used to withdraw the chloroform from the extraction vessel to the cuvette for measurement at 485 nm. Boiteux's method described a further two extractions of the sample with 15 ml aliquots of chloroform, with the final volume being made up to 50 ml. Our brief investigation showed that a single extraction (using the alkyl dimethylamine betaine) recovered a reproducible *ca.* 90% of the complex, making the other two extractions unnecessary (Table 6.2). Carrying out only a single extraction also reduced the volume of chloroform required from 50 ml to 15 ml which, considering the hazardous nature of the solvent, was considered advantageous. It was therefore recommended that the laboratories consider a similar approach for their analyses.

Table 6.2 Effect of repeat extractions for Orange II method

Replicate	Absorbance		
	1st Extraction	2nd Extraction	3rd Extraction
1	271	58	23
2	293	59	20
3	270	41	19
Mean blank corrected absorbance	258	33	0.7
Mean removal of colour into solvent (%)	**88.4**	**11.3**	**0.3**

A calibration between 0 and 10 mg l^{-1} was carried out using an alkyldimethyl betaine and comparisons performed using an amidobetaine and a sulpho hydroxy betaine (5 mg l^{-1}). Relative performances are presented in Table 6.3.

Table 6.3 Response of Orange II method to different amphoteric surfactants

Substance	Response (Abs/ppm)	Response (Abs/mmole)
Amidobetaine	0.250	0.090
Alkyl dimethyl betaine	0.344	0.101
Sulpho hydroxy betaine	0.058	0.025

Results showed that the sensitivity of the method to the sulpho hydroxy betaine was much reduced compared with that for the alkyl dimethylamine betaine and amidobetaine (which was in line with that obtained by Boiteux). The lack of sensitivity was attributed to the requirement to protonate the sulphoxy group before the complex could be extracted. This would occur at a much lower pH than that of an alkyl dimethyl- or amido betaines. Doubling the acid strength in the sample (pH <1), failed to improve the extraction efficiency significantly. It was therefore decided to use an alkyl dimethyl betaine and an amido betaine for the purposes of the study. These make up approximately 88% of the commercial market share in amphoteric surfactants (Europlus s.a. 1997).

6.3.3 Adsorption properties of cationics and analysis in the presence of silica

Two tests were performed was to test the adsorption properties of cationic surfactants onto the glass test vessels and silica added to alleviate the potential toxicity. Three replicates of 1 and 10 mg l^{-1} DODMAC (50 ml) were spiked into borosilicate glass vessels and the dissolved concentration determined after one and ten days (Table 6.4).

Table 6.4 Dissolved concentration of DODMAC in glass vessel with time

	Time			
	0	**5 mins**	**1 day**	**10 days**
DODMAC (mg l^{-1})	1	0.76	0.73	0.44
DODMAC (mg l^{-1})	10	10	10	9.4

Results show that there is significant adsorption of the cationic surfactant to glass, particularly at lower concentrations. In order to overcome this affinity, either the glassware has to be conditioned prior to use (such procedures normally accompany analytical methodologies), or the complexation/extractions have to be carried out within the test vessel.

In order to test the recovery of surfactant (CTAB) from samples containing silica (50-150 mesh, Merck Ltd), for each sample (15 ml) a 500 times excess of silica was added over the surfactant concentration (mg C l^{-1}). For CTAB at 1 mg l^{-1} surfactant = 0.63 mg C l^{-1} x 500 = 315 mg silica l^{-1}. Three replicates with and without silica were analysed using the above method. Table 6.5 shows the recoveries.

Table 6.5 Relative recoveries of CTAB with and without addition of silica

Treatment	**Replicate**	**CTAB added (mg l^{-1})**	**CTAB recovered (mg l^{-1})**	**Mean (mg l^{-1})**	**s.d.**
WITH silica	1	1.00	0.97		
	2	1.00	1.01	0.99	0.02
	3	1.00	0.98		
WITHOUT silica	1	1.00	0.94		
	2	1.00	1.00	0.98	0.040
	3	1.00	1.01		

The above data show that the complexation/extraction procedure is capable of recovering all the cationic surfactant adsorbed to the silica, thus allowing the entire contents of the test vessel to be sacrificed for each determination. This ensures that there is no bias associated with the analytical stage due to adsorption to the silica or vessel walls.

Conducting the complexation/extraction within the test vessel requires the conical flask to be sealed during the extraction process. This is best achieved by using flasks with ground glass 'quick fit' stoppers. Other methods, such as using bungs (provided that it can be demonstrated that there is no adsorption of analyte onto their surface), may also be used.

7. BIODEGRADATION TEST GUIDELINES

This section describes the methodology, based on OECD Die-Away Test (1976), used by the laboratories to carry out the primary biodegradation tests,

7.1 Introduction

7.1.1 Range of application

The method described here is limited to the examination of surfactants in the pure, or largely pure, state which are soluble in water (solubility of at least 10 mg l^{-1}) and which react positively in one or other of the semi-specific colorimetric tests described in Section 8. Surfactants present in commercial detergent formulations would first have to be separated from other constituents by e.g. extraction, ion exchange, etc. before analysis. Similarly, environmental samples would have to be subjected to separation procedures, for removal of any interfering substances that react with the colorimetric reagents, before application of the colorimetric methods for determination of individual classes of surfactants.

Note: This separation procedure will not be necessary for these tests as they will not contain other constituents.

7.1.2 Interferences

Some chemicals, such as strong alkalis, toxic metals, bactericides, organic solvents, in solution or in the air, can hinder the activity of micro-organisms. This may delay the bio-degradative process or influence the final results. Surfactants themselves, especially cationic surfactants may also inhibit the activity of micro-organisms when they are present at a sufficiently high concentration. Methods to overcome this potential problem are discussed in Section 8.2.

7.2 Principle

A pre-determined amount of the test surfactant is dissolved in an inorganic nutrient medium 7.3.2), providing a concentration of 5±0.5 mg l^{-1} of the surfactant. The solution is inoculated to give a relatively low density of a mixed population of aerobic micro-organisms. The mixture is aerated at 25± 1 ^{o}C until analysed, with samples taken over time to show that the concentration of surfactant falls to a constant level, or until 19 days have passed. The surfactants are determined by the appropriate semi-specific colorimetric test and the procedure is checked by simultaneously testing two anionic standard (or reference) surfactants, of known high and low biodegradability.

7.3 Reagents

7.3.1 Deionised or distilled water

- To be free from toxic substances (in particular copper) for general use as a solvent.

7.3.2 Inorganic nutrient/buffer solution

To about 900 ml of water (7.3.1) add l ml of each of the following solutions (a) to (d) and make up to 1 litre with water (7.3.1): (Use AR grade chemicals):

(a)	potassium dihydrogen phosphate (KH_2PO_4)	8.5 g
	dipotassium hydrogen phosphate (K_2HPO_4)	21.75 g
	disodium hydrogen phosphate. dihydrate (Na_2 $HPO_4.2H_2O$)	33.4 g
	ammonium chloride (NH_4Cl)	1.7 g
	Water (6.3.1) to	1000 ml
(b)	Magnesium sulphate heptahydrate (Mg $SO_4.7H_2O$)	22.5 g
	Water (7.3.1) to	1000 ml
(c)	Anhydrous calcium chloride ($CaCl_2$)	27.5 g
	Water (7.3.1) to	1000 ml
(d)	Ferric chloride hexahydrate ($FeCl_3.6H_20$)	0.25 g
	Water (7.3.1) to	1000 ml

This last solution is freshly prepared immediately before use.

7.3.3 Mercuric chloride solution

1% $HgCl_2$ in water (7.3.I).

7.4 Biodegradability standard substances

7.4.1 Soft anionic standard

For this ring test di-iso-octylsulphosuccinate sodium salt has been selected as the soft reference material. In a previous ring test (EU 4697, 1999), the succinate was shown to degrade by an overall average of 72% (9.5% s.d.) by normal inocula and by 87% (4.1% s.d.) by pre-exposed inocula, in an ultimate, ready biodegradability test after 28 days using CO_2 headspace analysis.

7.4.2 Hard anionic standard

A poorly degradable alkyl benzene sulphonate of the branched tetrapropylene benzene sulphonate type (TBS) is also required to establish that the test is not too lenient. Under the conditions of this test the biodegradability of this product is 0-35%.

7.5 Preparation of samples

7.5.1 Preparation of stock solutions of standard surfactants and test surfactants

To ensure that the concentration of surfactants in the inoculated test solutions (M) is in the range of maximum accuracy for the assessment of biodegradability, it is necessary to prepare a stock solution of each surfactant. Prepare these solutions to contain about 1 g l^{-1} of the surfactant allowing for the percentage "active matter" in the supplied surfactant (for precautions in preparation, see 7.5.2). From these stock solutions it will be possible to further dilute them to the 5 mg l^{-1} test concentration (M).

7.5.2 Preparation and handling of solution of surfactants

Appropriate precautions should be taken when preparing and transferring solutions or samples containing surfactants due to their foaming and adsorptive properties.

Precautions to be taken could include:

a) pre-condition all glassware, especially when testing cationic surfactants, by rinsing with, and then discarding, a small volume of the sample;

b) prepare standard solutions of surfactant carefully with the production of as little foam as possible; allow any foam to dissipate before adding solvent to the volumetric mark. The foam can be collapsed by gently blowing laboratory air or nitrogen through a clean Pasteur pipette onto the foam;

c) take samples from graduated flasks and test vessels only when the foam has settled;

d) when aliquots are taken from bottles, the contents should first be mixed by means of a magnetic stirrer or by inverting slowly a few times;

e) ensure that surfactant solutions are transferred a minimum number of times.

Whatever precautions are taken, it should be subsequently demonstrated that the precautions are successful, i.e. that concentrations in the test media are in fact what are intended and that any loss of surfactant in transfers is negligible (i.e. by using control solutions with the surfactants present, but no inocula).

7.6 Inoculation

7.6.1 Inoculum

Take secondary effluent of good quality from an efficiently operated wastewater treatment plant receiving predominantly domestic sewage and keep the sample of effluent aerated between sampling and application. Before use, filter through a coarse filter, discarding the first 200 ml. Keep the filtrate aerobic and use on the day of collection.

7.6.2 Inoculation

The inoculum should be checked experimentally when the method is used for the first time and when any change is made in the nature or source of the inoculum, so that the amount of inoculum chosen gives at least 80% primary biodegradation of the soft anionic standard and 0-35% of the hard anionic standard. This requires a test employing the two standards and the use of various quantities of the inoculum in replicate tests. Generally, 0.5 ml filtered effluent per litre medium will be adequate, with a maximum of 5 ml l^{-1}.

7.7 Apparatus

– Erlenmeyer flask (conical flasks with necks which are not too narrow) of 2000 ml capacity;

  [Note: For Cationic surfactants, smaller vessels (250 ml) will have to be used if silica is employed so that the complete contents of the vessel can be extracted for each analysis - see Section 8].

  The flasks must be carefully cleaned, rinsed with alcohol and dried before use to avoid contamination with residues from previous tests: take special care with new flasks.

– Shaking machine to accommodate the flasks in an incubator or a constant temperature room at 25±1 ^{o}C.

7.8 Procedure

7.8.1 Preparation

Samples and standards are tested simultaneously in triplicate. To batches of 2000 ml of mineral nutrient solution (see Section 7.3.2) add sufficient stock solution to give a 5 mg l^{-1} test solution, see Section 7.5) plus the required amount of the effluent inoculum (as determined in Section 7.6). * Allow any foam on these solutions (M) to disperse (see Section 7.5.2.b) and then determine their surfactant content, in triplicate. The mean value of each set of these triplicates is taken as the initial concentration (Co) which must lie between 4.5 and 5.5 mg active surfactant per litre and which should be estimated to the nearest 0. 1 mg l^{-1}.

** Note: Also prepare control samples which contain the surfactant (at 5 mg l^{-1}) but not inoculum. These must be analysed at the same time periods as the test samples, and*

provide an indication of the stability of the test sample (e.g. likelihood of adsorption or abiotic degradation). Mercuric chloride at 100 mg/l should be added to each solution to inhibit biological activity.

7.8.2 Incubation

Pour 900 ml of each batch (M)* into the reaction flasks (Section 7.7) and plug the mouths with loose cotton wool in a manner which does not impede the exchange of gas between the flask and surrounding atmosphere. Place the flasks on the incubated shakers. Ensure that the flasks are shielded from light and that the temperature is maintained at 25±1 ^{0}C throughout the incubation period. The surrounding air should be free from pollutants and toxic materials. Repeat this procedure for the control samples, but do not add inocula.

** For the alkyl dimethyl quarternary ammonium chloride surfactant the entire contents of the test vessel will have to be extracted due to the addition of silica. The test therefore needs to be performed in 250 ml sealable conical flasks (ensure method of sealing does not adsorb analyte) using a volume of 50 ml test solution. The other cationic surfactant (cationic B), should not exhibit the same potential for toxicity and can be treated the same as the others.*

7.8.3 Sampling

Take samples (at least three replicates) of just sufficient volume for carrying out a single determination of the relevant surfactant on each sample. * Take samples on Day 0, 8 and 19 at least. ** The duration of the test shall not exceed 19 days. Do not remove unnecessarily large volumes of reaction mixtures; for anionic surfactants 10 to 20 ml is sufficient at the start, increasing to 100 ml for the last samples.

* *Again for the cationic surfactants containing silica, the entire vessel is extracted (see Section 7.7).*

** *For the hard and soft anionic standards, samples need to be taken after Day 14 to test if they meet the required level of biodegradability (Section 7.10).*

7.8.4 Taking samples and their treatment

Before taking a sample allow time for any foam in the test vessels to disperse (or see Section 7.5.2.b); then take the sample using "conditioned" glassware and a minimum of operations (Section 7.5.2). It is preferable to carry out the analysis within 3hr of collection; otherwise preserve the sample by adding mercuric chloride solution (Section 7.3.3) to give a concentration of 50 mg $HgCl_2$ l^{-1}. The analyst should ensure that $HgCl_2$ does not interfere in the subsequent analysis. For the cationic surfactants, the whole content of the vessel (including the added silica) needs to be extracted (see Section 8.2).

7.9 Calculation of results

The percentage biodegradation (D) of a sample surfactant is calculated using the following equation:

$$D_t = (C_0 - C_t) / C_o \times 100\%;$$

where: D_t = percentage degradation at time t;

C_0 = mean initial concentration of surfactant in the test solution (M);

C_t = concentration of surfactant in the test solution (M) at time t;

both expressed as mg "active" surfactant per litre.

The percentage degradation in a single test (D_e) is obtained from the above equation when $D_t = D_e$ and $C_t = C_e$, where C_e represents the concentration of surfactant at the plateau. The arithmetic mean of the triplicate values of D_e is taken as the percentage degradation, D, of the sample.

For samples giving degradation curves without a plateau, C_e is determined at the end of the test, i.e. on the 19th day.

Calculate the results to the nearest 0.1% but give the final value, D, to the nearest whole number; results ending in 0.5 are rounded down to the nearest whole number. Calculate the percentage biodegradation of the standards (D_{s1} and D_{s2}) in the same way using the corresponding values of C_0 and C_t for the soft and hard standards.

7.10 Validity of results

For the results to be valid, the soft standard must be degraded to a value greater than 80% within a 14 day incubation period, and the hard standard to not more than 35%. Failing either of these conditions, the whole test series must be repeated, using a more appropriate bacterial cell density.

Note. The value of 35% was adopted over 25 years ago; since then reports have suggested that the limit has increased, due to a higher degree of adaptation of the environmental microbial population to the branched chain TBS.

8. CHEMICAL ANALYSIS GUIDELINES

The following methodologies were recommended to the laboratories for use with the primary biodegradation testing.

8.1 Anionic surfactants

The recommended technique for MBAS determination of the anionic reference surfactants is the methylene blue active substances colorimetric method, which is well documented, e.g.

HMSO (1981) Analysis of surfactants in waters, wastewaters and sludges, 1981. Methods for the examination of waters and associated materials, SCA, Her Majesty's Stationery Office, London, ISBN 0 11 751605 8.

EC legislation on detergents Directive 82/243/EEC

OECD (1976) Proposed method for the determination of the biodegradability of surfactants used in synthetic detergents. Environment Directive. OECD Paris.

8.2 Cationic surfactants

The method to be employed for the determination of cationic surfactants is the disulphine blue method (DBAS), which is described in the following references:

Standards: T 73-280 and DIN 38 409 Teil 20

HMSO (1981) Analysis of surfactants in waters, wastewaters and sludges, 1981. Methods for the examination of waters and associated materials, SCA, Her Majesty's Stationery Office, London, ISBN 0 11 751605 8.

The analytical technique used for the validation exercise (Section 6) was based on that described in the UK 'SCA blue book' (HMSO 1981). Given that the experiments using cationic surfactants will contain silica, the whole contents of each experimental flask will need to be analysed. As a consequence we recommend a 40 ml sample volume (rather than 15 ml as per the 'blue book' method), with volumes of buffer, reagent and solvent scaled up accordingly.

8.2.1 Reagents

All reagents were of Analytical Reagent Quality and all glassware thoroughly cleaned and rinsed with distilled water prior to use.

Sodium acetate buffer: 115 g anhydrous sodium acetate + 35 ml glacial acetic acid in 1 litre DIW.

Disulphine blue solution: 0.16 g disulphine blue - CI 42045, (Patent Blue Violet, 70%, Sigma Ltd) dissolved in 18 ml DIW, 2 ml ethanol and diluted into 250 ml of DIW.

Chloroform: Analytical grade.

Silica: see section 6.3.3.

Standards: 0, 0.1, 0,5 1, 5 mg l^{-1} of test surfactant (as active substance-accounting for purity), diluted from 1000 mg l^{-1} stock.

8.2.2 Procedure

1. To each replicate of the test conical flask (containing 50 ml of sample), add:

   - 5 ml of the buffer;
   - 2 ml of the reagent;
   - 15 ml of chloroform.

2. Seal flask - (we used 'quick fit' conicals and therefore used ground glass stoppers, other materials would have to be checked for potential adsorption).

3. Shake vigorously for 2 minutes.

4. Allow phases to separate for 5 minutes.

5. Using a Pasteur pipette remove an aliquot of chloroform (bottom phase) and discard; repeat (this conditions the pipette).

6. Using the same pipette remove another aliquot of the chloroform layer and place in a clean 1 cm cuvette; discard and refill cuvette with chloroform extract.

7. Measure absorbance at 628 nm (against a blank chloroform sample).

   Note: It is essential that water present in the samples is kept out of the cuvette, otherwise it will adhere to the walls and cause erroneous readings.

8. Carry out calibration using the test chemicals, followed by analysis of the biodegradation test samples.

   Note: Calibrations will be required for each individual surfactant, due to slight differences in sensitivity.

9. After each measurement, rinse cuvette with methanol to remove any complex adsorbed to the glass walls.

10. Calculate concentrations in the test vessels by using the appropriate calibration and blanks measurements.

    Note: The DBAS method is quite a sensitive technique, and at 5 mg l^{-1} the extraction of 50 ml of sample may result in absorbances exceeding 1 absorbance unit. If after

performing a calibration this is the case, then dilution of the sample will be required prior to analysis. This may be achieved by, for example, making up the sample volume in the test vessel to 200 ml with distilled or deionised water; then adding the reagents, extracting, and taking into account the dilution when calculating the concentration. To check this, it may be advisable to add an extra replicate into the test and use it as a 'sighter' to achieve the correct dilution for other samples. At the end of the experiment, when the surfactant has been degraded, such dilutions may not be necessary.

8.3 Amphoteric surfactants

It has been stated that in principle the DBAS method can be used to determine amphoteric surfactants (Europlus 1997), provided the surfactants are in their cationic form. This however, requires the pH to be reduced to an appropriate level prior to analysis. At the ambient pH used in the DBAS method (pH 4.7 - acetate buffer) none of the alkyl dimethylamine betaine or coco-amidopropyl dimethyl hydroxysulpho-betaine was extracted. Reduction in pH to 3.0 or below severely interfered with the complexation/extraction procedure and failed to improve the recoveries. In the absence of any literature data or published methodologies, and without time or funds to further the investigation, the DBAS method was not recommended for the determination of amphoteric surfactants as part of this ring test.

The method specifically developed to determine amphoteric surfactants is the Orange II method. The original method described by Boiteux used 100 ml samples, but it is recommended that this be scaled down to around 40 ml for practical reasons. The volumes of reagents were therefore also scaled down accordingly.

8.3.1 Reagents

All reagents were of Analytical Reagent quality and all glassware thoroughly cleaned and rinsed with distilled water prior to use.

Sulphuric acid: An 18N solution of sulphuric acid was prepared by slowly and gently adding concentrated sulphuric acid (100 ml) to distilled or deionised water 100 ml).

Note: - great care must be taken as the reaction of concentrated sulphuric acid with water is an extremely exothermic one, producing a large amount of heat.

Orange II Reagent: 1 g of Orange (II) (Sigma Ltd, 95% dye content) dissolved in 1 litre of deionised or distilled water containing 0.1 M sodium chloride (5.9 g).

Chloroform: Analytical Reagent grade.

Standards: 0, 0.1, 0,5 1, 5 mg l^{-1} of test surfactant (as active substance - accounting for purity), diluted from 1000 mg l^{-1} stock.

8.3.2 Procedure

1. Place a 40 ml sample in a suitable extraction vessel and add 0.8 ml of the sulphuric acid solution, 0.8 ml of the Orange (II) reagent and 7.5 ml of chloroform.

2. The extraction vessel was then shaken vigorously for 2 minutes, and the two layers left to separate.

3. A clean Pasteur pipette was used to pipette off an aliquot of the bottom layer containing the chloroform extract which was discarded (this conditions the pipette). This was repeated, then the third aliquot placed in a clean 1 cm cuvette.

   Note: It is essential that water present in the samples is kept out of the cuvette, otherwise it will adhere to the walls and cause erroneous readings.

4. Measure absorbance at 450 nm (against a chloroform blank).

5. Carry out calibration using the test chemicals, followed by analysis of the biodegradation test samples.

   Note: Calibrations will be required for each individual surfactant due to slight differences in sensitivity.

6. After each measurement, rinse cuvette with methanol to remove any complex adhered to the glass walls.

7. Calculate concentrations in the test vessels by using the appropriate calibration and blanks measurements.

9. RESULTS

9.1 Chemical analysis methodologies – general comments

An assessment of the test procedures was included as one of the objectives of the exercise, particularly as the analytical methodologies for the cationic and amphoteric surfactants are not necessarily routinely employed by the laboratories. The analytical results received from the laboratories showed that the two methods (disulphine blue and Orange II), performed adequately for the primary biodegradation test, with respect to both the limits of detection and the precision. There were, however, a few comments made by the laboratories regarding the analytical techniques that are worth noting:

Laboratory number 3 considered that 2 or 3 additions of solvent were required in order to reduce errors associated with using a relatively small volume of solvent per extraction (e.g. 7.5ml Orange II, 15ml for disulphine blue), which may lead to significant evaporation. Although there is potential for evaporation to occur, preliminary tests using both techniques (Section 6) indicated that only a single extraction set was required for the Orange II method. The SCA 'Blue Book' method also only specifies a single extraction step for the determination of cationic surfactants using the disulphine blue method. Increasing the number of extraction steps serves to dilute the extracted colour, and so degrade the limit of detection. It also offers the possibility of increased risk of contamination and makes the method more time-consuming and hence more expensive to perform. Taking into account the toxic nature of chloroform, it would seem prudent to keep the volume used to the minimum provided the analytical performance is not compromised.

It was also noted that the use of a 15ml solvent volume for the disulphine blue method resulted in absorbances very near to, or exceeding the top of the analytical range. For future analysis a slightly greater volume of solvent may need to be specified to remedy this (e.g. 20 or 25 ml per extraction). Although again, due to the toxic nature of the chloroform solvent used, it is in the interest of analyst's safety to keep the volumes used to a minimum.

Laboratory number 4 found difficulty obtaining reproducible results using both methodologies. They noted that the extinction decreased with time in the photometer and the analysis was hampered by water droplets being carried over into the analytical cuvette with the solvent. From our experience the transfer of the solvent to the cuvette is a very operator-sensitive process. Unless carried out in a very careful manner we found that pipetting the solvent resulted in air bubbles becoming entrained into the solvent placed into the photometer's cuvette. If this occurred, then the extinction decreased as the bubbles cleared. This problem could be overcome by using a positive displacement of the air inside the pipette as the tip was pushed down through the water layer into the solvent, this ensured no water entered the tip of the pipette. Once the solvent had been withdrawn it was allowed to run into the cuvette under gravity rather than by applying positive pressure. Laboratory 4 did state however, that the two phases were only allowed to stand and separate for two minutes before removal of the solvent. From our experience this may not be a sufficient length of time, and is dependent on the type and concentration of sample determined. A standing time of 5 minutes may provide a better separation, although it is important to keep these times constant to ensure that any changes in absorbances that occur with time are normalised. The laboratory also changed the volumes of the reagents slightly (50ml of sample, 1 ml of

Orange II reagent, 1 ml of sulphuric acid and 10ml of chloroform) without compromising the analytical quality.

It was also noted by laboratory number 4 that in the case of Cationic B (diesterquat), that a reproducible straight line calibration could not be obtained for the initial samples. However, a satisfactory calibration was reported at the end of the experiment, with 30% increase in the sensitivity. No other laboratory reported this problem, although the disulphine blue method has been largely applied to di- and tri- methyl alkyl ammonium cationic surfactants, rather than the relatively new ester-quats. Given the hydrolytic nature of the ester-quats it is possible that under certain conditions that the colour may not be stable.

Laboratories 1, 2, 5 and 6 had no comments regarding the analysis.

9.2 Biodegradation results - general

Test conditions and the mean values for the percentage removal of the relevant surfactant, and derived statistical data, obtained for the two reference substances and the 4 test surfactants are given in Tables A1 to A7, Appendix A. Detailed raw data and calculations for the six laboratories are given in Tables B1 to B6 in Appendix B. Wherever possible, values reported were checked with the laboratories. In some cases, a few errors were found and data changes resulted from rounding values to two significant figures. The same results are presented in Tables 9.1 to 9.6 in the main text, although here each Table gives the test results for one surfactant all those laboratories participants. Table 9.7 gives a summary by presenting the overall mean values and associated statistical data for each surfactant using inoculated test medium. Table 9.8 presents the smaller number of results obtained using uninoculated medium.

Results with inocula can be assumed to indicate the sum of biotic and abiotic or “total” removal. Those obtained without inocula are usually taken to indicate abiotic removal by physical processes (e.g. adsorption, volatilisation etc) and chemical degradation (photolysis and hydrolysis). However, it has been reported that some chemicals are biodegraded in uninoculated test-media (by finding degradation in the media but not when $HgCl_2$ is added) and has been attributed to the presence of micro-organisms in deionised water. Such water can contain around 10^3 cells/ml.

It will be seen in the Tables that four laboratories tested all six surfactants, the fifth studied the two reference surfactants and two other surfactants and the sixth studied all except cationic A. One participant returned data obtained after 19 days incubation only, while others reported results for 19 days and for 8 and/or 14 days.

Results using uninoculated medium were reported by only four participants, three of whom added mercuric chloride to inhibit bacterial action.

9.3 “Total” removal – reference surfactants

With one exception, the values for the removal of the soft anionic surfactant after 14 to 19 days were all well above the “pass” value of 80%, with a mean value of 98%, +/- 15% (n=5). The sixth laboratory recorded a value of only 70%, +/- 3, at days 8 and 14, with this result included the mean was 94%, +/- 11.6 (n=6). Intermediate results indicated that the “pass”

value has been reached in 8 days (1 laboratory), 14 days (3 laboratories) or 19 days (1 laboratory). The percentage rate of removal shows that the inocula from these five laboratories contained sufficiently high active populations of micro-organisms. The fact that the inoculum from laboratory number 6 achieved only 70% removal of the soft surfactant after both 8 and 14 days is not unprecedented. When this happens, the test should be repeated using a higher concentration of inoculum. In such a case only 1 ml effluent per litre had been used and it is to be expected that increasing this to, e.g. 5 ml would have produced a higher removal rate; but in this case due to lack of time, a repeat test was not possible. However, the results with cationic B and amphoterics A and B (Tables 9.4 and 9.7), showed that this inoculum was able to remove over 95% of these three surfactants (cationic A was not tested), after 19 days.

The hard anionic surfactant behaved problematically. Although the mean was as low as 27%, the standard deviation was high, at +/- 34%, with a high coefficient of variation of 125%. Four individual values were low (<13%), including that of laboratory number 6, with the others very much higher (at 70%), than the expected value of around 35% (Table 9.7). The validity criterion for this value is 25-35% after 14 days and is set at this value to try to ensure that the inoculum is not too “over active” so that it might “pass” surfactants which will not biodegrade readily in waste water treatment and in the environment generally. It should be noted, however that since the hard anionic surfactant was first introduced into the environment about 30 years ago, it could be expected that environmental micro-organisms had become more acclimatised to the branch-chained alkyl benzene sulphonates.

In the four comparisons possible (laboratories 1 to 4), there is no obvious correlation between the ability of the inocula to remove the hard standard and their ability to remove cationic surfactant A, which is not removed uniformly well by the four inocula. That is, the two low values for the hard standard, 0, 13% relate to the low (9%) and the high (89%) removal of cationic surfactant A, while the two high values (70%) relate to one high (98%) and one low (28%) value, respectively. Thus, the relatively high activity of some inocula towards the hard standard is not considered to bias the other results.

9.4 “Total” removal – amphoterics and cationics

The removal of cationic B and the two amphoterics by all six laboratories was, with one exception, well above the “pass” level of 80% (Table 9.7). The exception was laboratory 5 (see also Table A.6) which, with 71% failed to pass the cationic B surfactant and caused the coefficient of variation to be 11%, compared with 1.6% and 4.5% for the two amphoterics respectively.

The results with cationic A (mean 63% +/- 35%) were very varied with a coefficient of variation as high as 56%; there were two “passes” out of four. All four laboratories used SiO_2 in the test for this surfactant; laboratory number 1 reported a removal of 35% in the presence of SiO_2 and 9% in its absence.

9.5 Abiotic removal

The original OECD Die Away tests for assessment of the primary biodegradation of anionic and non-ionic surfactants did not include controls to determine whether removal of the surfactant occurred by processes other than biological ones, i.e. abiotically. Presumably the

need for such controls had been shown to be unnecessary in investigations before adoption of the final test protocols, though a search of OECD files revealed no such investigation. The three laboratories that tested the standard anionics under “abiotic” conditions reported no removal (Table 9.8), the “total” removals were positive (98%, 96% and 70%) for the soft standard (Table 9.7).

There was no apparent pattern with the other four surfactants. No abiotic removal was found for the three laboratories that tested amphoteric B. For amphoteric A the same three laboratories reported no (0, 1.4%) or little (25%) removal abiotically, but the fourth (laboratory 5) reported complete removal (98%); the last laboratory did not add $HgCl_2$. Cationic B was found not to be degraded abiotically by two laboratories (nos. 4 and 6), while the other two reported 70% and 78% removal. The 70% value (lab no. 5) was obtained in the absence of the inhibitor, but the 78% value was obtained in its presence. Cationic A was removed to a high degree (70% and 94%) by the only two laboratories that tested it. The high values for the two cationics reported by laboratory 3 (namely 94% for A and 78% for B) were verified on repetition of the test. This laboratory suggested that mercuric chloride could have interfered in some way in the colorimetric test to give the apparent removal. However, on further investigation it was found that only a sub aliquot of the cationic A and B sample was taken for analysis due to absorbances being off scale of the colorimetric technique. It is therefore highly likely that adsorption to the vessel walls, particularly for the cationic A sample that is known to sorb strongly to surfaces, resulted in the apparent abiotic loss from solution.

In the 13 cases where a comparison can be made (for cationics and amphoterics), 12 indicate the same or lower value for the abiotic removal compared with the “total” removal. In the thirteenth case an improbable result was reported for cationic A by laboratory 4 of 70% for abiotic and only 28% for “total”. The most probable cause was experimental error.

In the environment, abiotic removal of substances may be brought about by a number of processes including physical removal (by adsorption to surfaces or volatilisation), or chemical degradation (by hydrolysis and/or photolysis). The test conditions will therefore be critical in controlling these types of mechanisms. In addition, activity of bacteria present in the uninoculated medium (if sterilisation has not been used), may also result in biodegradation in “abiotic” tests.

Table 9.1 Removal of Soft Anionic Surfactant

Lab No.	% Removal after					
	7-8 days		14 days		19 days	
	"Total"	"Abiotic"	"Total"	"Abiotic"	"Total"	"Abiotic"
1	98.6 (0) [0]	-	-	-	98.0 (0.1) [0.1]	-
2	-	-	99.1 (0.8) [0.8]	-	100 (0) [0]	-
3	-	-	-	-	97.6 (1.9) [2.0]	0
4	0	-	99.8 (0.2) [0.2]	-	99.1 (0.5) [0.5]	-
5	0	0	95.7 (0) [0]	0	-	-
6	70 (3.0) [4.2]	2.2	70.1 (3.2) [4.6]	-	-	2.2

Values () are standard deviations (n=5)
Values [] are coefficients of variation (%)
"Total" = inoculated "Abiotic" = uninoculated.

Table 9.2 Removal of Hard Anionic Surfactant

Lab No.	% Removal after					
	7-8 days		14 days		19 days	
	"Total"	"Abiotic"	"Total"	"Abiotic"	"Total"	"Abiotic"
1	4.1 (5.8) [147]	-	-	-	0	-
2	-	-	7.7 (1.5) [19]	-	13.2 (1.8) [14]	-
3	-	-	-	-	69.9 (11) [15]	0
4	0	-	(a) 58.5 (8.5) [15] (b) 39.9 (4.9) [12]	-	(a) 70.3 (7.5) [11] (b) 69.3 (3.7) [5.3]	-
5	0	0.7	1.3 (0.9) [70]	0	-	-
6	5.5 (2.5) [42]	2.7	9.4 (7) [77]	-	-	4.8

Values () are standard deviations (n=3)
Values [] are coefficients of variation (%)
"Total" = inoculated "Abiotic" = uninoculated.
(a) and (b) denote separate experiments.

Table 9.3 Removal of Cationic A Surfactant

Lab No.	% Removal after			
	7-8 days		19 days	
	"Total"	"Abiotic"	"Total"	"Abiotic"
1	(d) 4.8 (4.2) [87] (e) 31.0 (3.5) [11]	-	(d) 9.2 (3.9) [42] (e) 35.0 (8.1) [23]	-
2	29.2 (2.3) [7.9]	-	88.6 (0.9) [10]	-
3	-	-	98.0 (0.5) [0.5]	93.8
4	0	34	27.5 (13.5) [49]	70

Values () are standard deviations (n=3)
Values [] are coefficients of variation (%)
"Total" = inoculated "Abiotic" = uninoculated.
(d) = without SiO_2 (e) = with SiO_2

Table 9.4 Removal of Cationic B Surfactant

Lab No.	% Removal after			
	7-8 days		19 days	
	"Total"	"Abiotic"	"Total"	"Abiotic"
1	96.9 (0.4) [0.4]	-	99.3 (0.2) [0.2]	-
2	86.1 (0.9) [1.0]	-	96.0 (0.8) [0.8]	-
3	-	-	95.2 (1.3) [1.4]	78.1
4	(a) 91.4 (3.9) [4.3] (b) 94.4 (1.0) [1.0]	0	(a) 94.7 (6.9) [7.3] (b) 95.5 (0.3) [0.3]	0
5	48.5 ((9.2) [19]	50.4 (7.8) [16]	70.4 (7.8) [11]	70.1 (1.5) [2.2]
6	93.9 (1) [1]	0.7	94.5 (1.2) [1.3]	1.4

Values () are standard deviations (n=3)
Values [ ] are coefficients of variation (%)
"Total" = inoculated "Abiotic" = uninoculated.
(a) and (b) denote separate experiments.

Table 9.5 Removal of Amphoteric Surfactant A

Lab No.	% Removal after			
	7-8 days		19 days	
	"Total"	"Abiotic"	"Total"	"Abiotic"
1	50.4 (41) [80]	-	98.7 (0.2) [0.2]	-
2	95.7 (0.9) [0.7]	-	95.6 (0.6) [0.6]	-
3	-	-	100 (0) [0]	24.7
4	(a) 94.0 (0.9) [0.9] (b) 93.8 (1.7) [1.8]	0	(a) 100 (0) [0] (b) 100 (0) [0]	0
5	96.8 (2.3) [2.4]	98.0 (0) [0]	98.1 (0) [0]	98.0 (0) [0]
6	84.8 (2.1) [2.5]	0.5	100 (-) [-]	1.4

Values () are standard deviations
Values [ ] are coefficients of variation (%)
"Total" = inoculated "Abiotic" = uninoculated.
(a) and (b) denote separate experiments.

Table 9.6 Removal of Amphoteric Surfactant B

Lab No.	% Removal after			
	7-8 days		19 days	
	"Total"	"Abiotic"	"Total"	"Abiotic"
1	97.5 (1.8) [1.8]	-	98.8 (2.6) [2.6]	-
2	95.8 (0.9) [0.9]	-	96.9 (0.2) [0.2]	-
3	-	-	89.3 (4.6) [5.2]	0
4	(a) 94.2 (1.7) [1.8] (b) 94.3 (2.3) [2.4]	14	(a) 98.8 (0.4) [0.4] (b) 97.4 (1.2) [1.2]	3
6	85 (1) [1.2]	0	99.9 (0.1) [0.1]	1

Values () are standard deviations
Values [ ] are coefficients of variation (%)
"Total" = inoculated "Abiotic" = uninoculated.
(a) and (b) denote separate experiments.

An overall assessment of the degradation of each test surfactant is given in Table 9.7

Table 9.7 Summary of % Removals of six surfactants after 19 days in the OECD Die Away Test

Laboratory No.	% Removal of surfactant after 19 days					
	Soft Anionic	Hard Anionic	Cationic* A	Cationic B	Amphoteric A	Amphoteric B
1	98	0	35 (9)**	99	99	99
2	100	13	89	96	96	97
3	98	70	98	95	100	89
4	99	70	28	95	100	98
5	96	1	-	71	98	-
6	70	9	-	95	100	100
Mean	93.5	27.2	62.5	91.8	98.8	96.6
Mean (rounded)	94	27	63	92	99	97
s.d.	11.6	33.7	35.2	10.3	1.6	4.5
c.v. (%)	12.3	125	55.8	11.0	1.6	4.5
n	6	6	4	6	6	5

* SiO_2 added. ** without SiO_2.

Results for abiotic removal of the test surfactants are provided in Table 9.8.

Table 9.8 "Abiotic" removal of surfactants

Lab No.	% Removal after											
	8 d	19d	8d	19d	8d	19d	8d	19d	8d	19d	8d	19d
	Soft Anionic		Hard Anionic		Cationic A		Cationic B		Amphoteric A		Amphoteric B	
3	-	0	-	0	-	94**	-	78**	-	25	-	0
4	-	-	-	-	34	70	11	0	0	0	14	3
5*	0	0 (14d)	0	0.7 (14d)	-	-	50	70	98	98	-	-
6	2.2	2.2	2.7	4.8	-	-	0.7	1.4	0.5	1.4	0	1.0

* no $HgCl_2$ added.
** same values on repetition.

10. CONCLUSIONS

10.1 Analytical methodology

From the results it appears that on the whole the analytical techniques specified in the protocol were adequate to monitor the disappearance of the amphoteric and cationic surfactants during the primary biodegradation tests. One laboratory reported difficulties with obtaining a stable absorbance in the colorimeter, the cause of which was probably air bubbles entrained into the solvent during pipetting. By adhering to a careful, set procedure this problem can be eliminated.

The stability of diester-quat cationic surfactants was questioned by one laboratory that had difficulty in obtaining reproducible calibrations for the cationic B sample. This class of compound is known to be hydrolytically unstable and this may influence the stability of the coloured complex they form with the reagent. Most previous work utilising the disulphine blue method has been to determine dialkyl-quats rather than diester quats that now represent 99% of the market share of cationic surfactants (Europlus 1997). Further investigation of the response and stability of diester-quat surfactants to the disulphine blue method is recommended.

The Orange II method for amphoteric surfactants is relatively new and as such has not been as rigorously tested as the disulphine blue technique for cationics. Although it was shown to perform well with the two test surfactants (a dimethylamine betaine and a coco-amido betaine) which comprise an estimated 88% of the current market (Europlus 1997), it did not respond well to a sulphohydroxy betaine. With an increasing number of different surfactants on the market, further assessment of the methodology is recommended, to test its response to other classes of amphoteric surfactants such as glycinates etc.

The lack of information on the response of both techniques (Orange II and disulphine blue) to an ever increasing range of amphoteric and cationic surfactants was originally highlighted by the industry.

Chloroform is a toxic solvent and a suspected carcinogen, in the longer term it may be advised to consider other solvents (e.g. dichloromethane) as a replacement.

10.2 Primary biodegradation tests

The disappearance of disulphine blue or Orange II activity in the OECD Die Away test does not imply that biodegradation has occurred, hence results are expressed as percentage removal rather than percentage biodegradation. The results with the soft and hard anionic standards may be taken as being caused by microbial action alone since in the absence of an inoculum no removal took place. Adsorption on to the suspended solids derived from the inoculum could not have accounted for the removal of the soft surfactant since the concentration of suspended solids was extremely low. Also, if adsorption were important, the hard standard would also have been removed in the presence of the inoculum.

In addition, the values often obtained for % removal of the soft standard shows that the inocula were suitably active; the value for % removal of the hard standard, though somewhat higher than stipulated, also satisfies the validity criterion ruling out “over-active” inocula.

The abiotic tests were undertaken because the diesterquat (cationic B) was thought to hydrolyse fairly rapidly in aqueous solutions. If they had not been performed the conclusions from the inoculated tests would have been as follows:

- The soft and hard anionic surfactants behaved as expected.
- Cationic A was removed adequately (passing) by 2 out of the 4 laboratories.
- Cationic B was similarly removed by 5 out of 6 laboratories.
- Both the amphoterics A and B were adequately removed with 6 out of 6 and 5 out of 5 passes respectively.

However, the results of the abiotic tests confuse the situation for the cationics and amphoterics. For cationic A only two comparisons are available and they conflict; one suggests that the removal is abiotic only, while the other seems impossible, with the “total” being lower than the abiotic. Further discussion with laboratory 3 however, revealed that sub aliquots of the samples were taken for analysis; hence adsorption of the surfactant to the vessel walls would result in over estimation of the abiotic removal. The four comparisons for cationic B also conflict. Two suggest biological removal and two indicate that abiotic removal played the major role. Overall these tests highlight the operational difficulties in testing cationic surfactants that show a strong tendency to sorb to surfaces. With the amphoterics the results are clearer. Amphoteric B clearly degrades biologically in all three laboratories making the comparison, while amphoteric A was biologically removed in three of the four laboratories in which it was tested.

The use of silica as a method for reducing the toxicity of dialkylquats, such as test surfactant cationic A may, also lead to a reduction in the bioavailability of the compound to microbial degradation. If this were to be the case, then the addition of silica would obviously bias the results against this class of surfactants.

It is concluded that amphoteric B is biodegraded and reaches the pass level while amphoteric A is probably also adequately biodegraded. The evidence for cationic B is less convincing than that for cationic A. The latter showed 98% abiotic removal in one laboratory, but the test was not inhibited; while for cationic B a high value (70%) was similarly obtained and a further laboratory reported 78% in the presence of the inhibitor. There is insufficient evidence concerning cationic A and this is conflicting, so that no conclusion may be reported.

It is sometimes argued that providing a substance is removed in the test it does not matter whether it is removed by biological or abiotic mechanisms, this view is incorrect. Time is an important factor in these removals. The die away test lasts up to 19 days but in the activated sludge process the hydraulic retention time (HRT) is usually around 6 hours, (i.e. the wastewater is in the aeration tank for only 6 hours). Although the biological process is accelerated in the aeration tank, since the concentration of the micro-organisms in the sludge is around 1000-fold higher than in the die away test, the rate of physico-chemical hydrolysis or of volatilisation of volatile test substances is not enhanced. To estimate the rate of physico-chemical removal in the activated sludge process, determinations should be made in the die-away test over the first few hours, rather than after 14 and 19 days as in the normal test.

11. RECOMMENDATIONS

The two semi-specific tests can be used to determine the biodegradability and/or removal of cationic and amphoteric surfactants with the following provisos:

- In view of some of the analytical difficulties encountered by the participating laboratories it is recommended that adequately detailed guidance notes are offered to support the Directive.

- Perform the analytical procedure in the presence of silica and investigate its use in the tests for potentially toxic dialkyl-quat cationic surfactants (e.g. cationic A test surfactant) that the silica serves to reduce toxicity, but conversely, does not reduce the bioavailability of the surfactant to the test organisms, thus biasing the results.

- Given the improbable results obtained for cationic A, especially in the presence of $HgCl_2$, it would be advisable to check potential interferences caused by mercuric chloride on the disulphine blue and Orange II tests.

- When silica is used, emphasise the need for setting up vessels for each time interval and that all the reaction mixture (in each batch of reaction mixture) must be taken for DBAS analysis.

- Further work needs to be undertaken to clarify whether surfactants like cationics A and B are removed/degraded by physico-chemical processes – e.g. hydrolysis.

- Consider investigating a possible replacement for chloroform as the extraction solvent for the disulphine blue and Orange II analytical techniques.

- Consider testing the disulphine blue method for a broader range of diesterquat cationic surfactants.

- Make clear in the Directive that the tests are not specifically for primary biodegradability, but for ‘removal' or ‘total degradability’, as for the diesterquat cationic and potentially some amphoteric surfactants, abiotic degradation through hydrolysis and potentially photolysis are significant degradation pathways.

- Ring Tests

  i) Where tests are to be tackled by means of a ring test and the analytical method is “new”/”problematic”, 2 or 3 laboratories should be authorised to ‘pilot’ the method to reveal and overcome potential pitfalls.

  ii) Six laboratories are insufficient for getting acceptable results, especially when the required protocol is not followed.

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APPENDIX A DATA PRESENTED FOR EACH INDIVIDUAL LABORATORY

Table A.1 Summary of the test conditions

Lab No.	pH	Temp (^oC)	Type of sewage	Effluent suspended solids (mg dry solids/l)	Inoculum quantity (ml l^{-1} of medium)
1	6.9	25	Secondary effluent	0.6	1.0
2	7.1	22	30% industrial 70% domestic	0.15	
3	7.1	25	40% industrial 60% domestic		1.0
4	7.4	22-25	25% industrial 75% domestic	0.82	1.5
5	nd	25	<20% industrial >80% domestic	<10.0	5.0 (filtered)
6	7.4	25	Mainly domestic	-	1.0

Table A.2 Removal of six surfactants (as such and as DOC) in the OECD Die-Away Test

Laboratory Number 1

Surfactant	% Removal		
	After 7d	After 19d	
	Surfactant	Surfactant	DOC
Soft Anionic	98.6 (0) [0]	98.0 (0.1) [0.1]	75
Hard Anionic	4.1 (5.8 [141]	0	60
Cationic A – no SiO2 added	4.8 (4.2) [87]	9.2 (3.9) [42]	0
Cationic A – with SiO_2	31.0 (3.5) [11]	35.0 (8.1) [23]	-
Cationic B	96.9 (0.4) [0.4]	99.3 (0.2) [0.2]	66
Amphoteric A	50.4 (41) [80]	98.7 (0.2) [0.2]	89
Amphoteric B	97.5 (1.8) [1.8]	98.8 (2.6) [2.6]	90

N = 3 for surfactant analysis, except hard anionic, n =2
N=1 for DOC analysis
( ) = standard deviation [ ] = coefficient of variation, %

Table A.3 Removal of six surfactants in the OECD Die-Away Test

Laboratory Number 2

Surfactant	% Removal		
	7days	14 days	19 days
Soft Anionic	-	99.1 (0.8) [0.8]	100 (0) [0]
Hard Anionic	-	7.7 (1.5) [19]	13.2 (1.8) [14]
Cationic A*	29.2 (2.3) [7.9]	-	88.6 (0.9) [0.9]
Cationic B	86.1 (0.9) [1.0]	-	96.0 (0.8) [0.8]
Amphoteric A	95.7 (0.6) [0.7]	-	95.6 (0.6) [0.6]
Amphoteric B	95.8 (0.9) [0.9]	-	96.9 (0.2) [0.2]

n = 3 ( ) = standard deviation [ ] = coefficient of variation, %
* Test carried with silica added

Table A.4 Removal of six surfactants in the OECD Die-Away test – with & without inoculation

Laboratory Number 3

Surfactant	**% Removal after 19 day**	
	Inoculated	Not inoculated
Soft Anionic	97.6 (1.9) [2.0]	0
Hard Anionic	69.9 (11) [15]	0
Cationic A + SiO_2	98.0 (0.5) [0.5]	93.8
Cationic B	95.2 (1.3) [1.4]	78.1
Amphoteric A	100 (0) [0]	24.7
Amphoteric B	89.3 (4.6) [5.2]	0

Inoculated n = 3; not inoculated n=1
( ) = standard deviation [ ] = coefficient of variation, %

Table A.5 Removal of six surfactants in the OECD Die-Away test with & without inoculation

Laboratory Number 4

Surfactant	**% Removal**		
	7days	14 days	19 days
Soft Anionic – inoculated	0	99.8 (0.2) [0.2]	99.1 (0.5) [0.5]
- uninoculated	-	-	-
Hard Anionic – inoculated	0	(a) 58.5 (8.5) [15]	(a) 70.3 (7.5) [10.7]
- uninoculated	-	(b) 39 (4.9) [12]	(b) 69.3 (3.7) [5.3]
		-	-
Cationic A - inoculated	0	-	27.5 (13.5) [49]
+ SiO_2 - uninoculated	34	-	70
Cationic B - inoculated	(a) 91.4 (3.9) [4.3]	-	(a) 94.7 (6.9) [7.3]
- uninoculated	(b) 94.4 (0.9) [1.8]		(b) 95.5 (0.3) [0.3]
	11	-	0
Amphoteric A – inoculated	(a) 94.0 (0.9) [1.7]	-	(a) 100 (0) [0]
- uninoculated	(b) 94.1 (0.9) [1.8]	-	(b) 100 (0) [0]
	0		0
Amphoteric B – inoculated	(a) 94.2 (1.7) [1.8]	-	(a) 98.8 (0.4) [0.4]
- uninoculated	(b) 94.3 (2.3) [2.4]		(b) 97.4 (1.2) [1.2]
	14	-	3

Inoculated n = 3; not inoculated, details to come
( ) = standard deviation [ ] = coefficient of variation, %
(a) and (b) denote separate experiments * = to come

Table A.6 Removal of four surfactants in the OECD Die-Away test – with & without inoculation

Laboratory Number 5

Surfactant	% Removal					
	7days		14 days		19 days	
	Inoculated	Not inoculated	Inoculated	Not inoculated	Inoculated	Not inoculated
Soft Anionic	0	0	95.7 (0) [0]	0	-	-
Hard Anionic	0	0.7	1.3 (0.9) [70]	0	-	-
Cationic B	48.5 (9.2) [1.9]	50.4 (7.8) [16]	-	-	70.5 (7.8) [11]	70.1 (1.5) [2.2]
Amphoteric A	96.8 (2.3) [2.4]	98.0 (0) [0]	-	-	98.1 (0) [0]	98.0 (0) [0]

n = 3
( ) = standard deviation [ ] = coefficient of variation, %

Table A.7 Removal of five surfactants in the OECD Die-Away test with & without inoculation

Laboratory Number 6

Surfactant	% Removal		
	8 days	14 days	19 days
Soft Anionic – inoculated	70.0 (3.0) [4.2]	70.1 (3.2) [4.6]	-
- uninoculated*	2.2	-	2.2
Hard Anionic – inoculated	5.5 (2.5) [42]	9.4 (7.0) [77]	-
- uninoculated*	2.7	-	4.8
Cationic B - inoculated	93.9 (1.0) [1.0]	-	94.5 (1.2) [1.3]
- uninoculated*	0.7	-	1.4
Amphoteric A – inoculated	85.0 (1.0) [1.2]	-	100 (0) [0]
- uninoculated*	0	-	1.4
Amphoteric B – inoculated	85.0 (1.0) [1.2]	-	99.9 (0.1) [0.1]
- uninoculated*	0	-	1.0

n = 3;
( ) = standard deviation [ ] = coefficient of variation, %
* = $HgCl_2$ added at 100 mg l^{-1}

APPENDIX B RESULTS FOR INDIVIDUAL LABORATORIES

Table B.1 Removal of six surfactants in the OECD Die-Away test – after 7 & 19 days

Laboratory Number 1

Surfactant	n	Concentration (mg l^{-1})			% Removal of surfactant after	
		Co	C7d	C19d	7d	19d
Soft Anionic	1	4.24	0.06	0.08	98.6	98.1
	2	4.26	0.06	0.09	98.6	97.9
	3	4.3	0.06	0.09	98.8	97.9
	mean				98.6	98.0
					(0) [0]	(0.1) [0.1]
Hard anionic	1	4.90	4.50	4.90	8.2	0
	2	4.70	5.22	5.42	0	0
	mean				4.1	0
					(5.8) [141]	
Cationic A (- SiO_2)	1	4.72	4.37	4.50	7.4	4.7
	2	4.87	5.07	4.30	0	11.7
	3	4.93	4.58	4.38	7.1	11.1
					4.8	9.2
+ SiO_2					(4.2) [87]	(3.9) [42]
	1					
	2				27.1	42.4
	3				32.0	36.1
	mean				33.9	26.4
					31.0	35.0
					(3.5) [11]	(8.1) [23]
Cationic B	1	4.65	0.14	0.04	97.0	99.1
	2	4.67	0.13	0.03	97.2	99.4
	3	4.63	0.16	0.03	96.5	99.4
	mean				96.9	99.3
					(0.4) [0.4]	(0.2) [0.2]
Amphoteric A	1	4.10	3.3	0.06	19.5	98.5
	2	4.70	0.2	0.06	97.5	98.7
	3	5.00	3.2	0.06	36.0	98.8
	mean				50.4	98.7
					(41) [80]	(0.2) [0.2]
Amphoteric B	1	4.1	0.1	0.06	97.6	95.5
	2	4.80	0.04	0.05	99.2	99.0
	3	4.70	0.2	0.05	95.7	98.9
	mean					98.8
					97.5	(2.6) [2.7]
					(1.8) [1.8]	

( ) = standard deviation [ ] = coefficient of variation, %

Table B.2 Removal of six surfactants in the OECD Die-Away test – after 8, 14 & 19 days

Laboratory Number 2

Surfactant	n	Concentration in (mg l^{-1})				% Removal		
		C_o	C_{8d}	C_{14d}	C_{19d}	8d	14d	19d
Soft Anionic	1	4.70	-	0.04	0	-	99.1	100
	2	4.72	-	0.03	0	-	99.4	100
	3	4.76	-	0.05	0	-	98.9	100
	mean						99.1	100
							(0.8)	
							[0.8]	
Hard Anionic	1	4.93	-	4.54	4.30	-	7.9	12.8
	2	4.95	-	4.50	4.20	-	9.1	15.2
	3	4.86	-	4.56	4.29	-	6.2	11.7
	mean						77	13.2
							(1.5) [19]	(1.8) [14]
Cationic A	1	4.66	3.20	-	0.49	31.3	-	89.5
	2	4.60	3.37	-	0.56	26.7	-	87.8
	3	4.62	3.25	-	0.53	29.6	-	88.5
	mean					29.2		88.6
						(2.3)		(0.9)
						[7.9]		[0.9]
Cationic B	1	4.95	0.69	-	0.18	86.1	-	96.4
	2	4.98	0.65	-	0.18	86.9	-	96.4
	3	4.94	0.73	-	0.24	85.1	-	95.1
	mean					86.1		96.0
						(0.9) [1]		(0.8)
								[0.8]
Amphoteric	1	4.78	0.18	-	0.19	96.2	-	96.0
A	2	4.78	0.24	-	0.21	95.0	-	95.6
	3	5.11	0.21	-	0.25	95.9	-	95.1
	mean					95.7		
						(0.6)		95.6
						[0.7]		(0.6)
								[0.6]
Amphoteric	1	4.45	0.19	-	0.14	95.7	-	96.9
B	2	4.39	0.14	-	0.13	96.8	-	97.0
	3	4.61	0.23	-	0.15	95.0	-	96.7
	mean					95.8	-	96.9
						(0.9)		(0.2)
						[0.9]		[0.2]

( ) = standard deviation [ ] = coefficient of variation, %

Table B.3 Removal of six surfactants in the OECD Die-Away test – (Total & Abiotic)

Laboratory Number 3

Surfactant	n	Concentration in (mg l^{-1})				% Removal	
		Inoculated		Not inoculated			
		C_o	C_{19d}	C_{0d}	C_{19d}	8d	14d
Soft Anionic	1	6.3	0.18	6.0	6.6	97.1	0
	2	5.8	0.10	-	-	98.3	-
	3	5.9	0.15	-	-	97.5	-
	mean					97.6 (1.9) [2.0]	0
Hard Anionic	1	6.5	2.3	5.5	5.9	64.6	0
	2	6.2	1.1	-	-	82.3	-
	3	5.1	1.9	-	-	62.7	-
	mean					69.9 (11) [15]	0
Cationic A (+ silica)	1	3.7	0.07	3.9	0.24	98.1	93.8
	2	4.5	0.07	-	-	98.4	-
	3	3.5	0.09	-	-	97.4	-
	mean					98.0 (0.5) [0.5]	93.8
Cationic B (- silica)	1	3.3	0.19	2.6	0.57	94.2	78.1
	2	3.2	0.17	-	-	94.7	-
	3	3.3	0.11	-	-	96.7	-
	mean					95.2 (1.3) [1.4]	78.1
Amphoteric A	1	5.5	-0.11	7.7	5.8	100	24.7
	2	3.9	–0.11	-	-	100	-
	3	6.0	–0.13	-	-	100	-
	mean					100	24.7*
Amphoteric B	1	2.8	0.44	3.9	5.5	84.3	0
	2	4.0	0.27	-	-	93.3	-
	3	3.4	0.33	-	-	90.3	-
	mean					89.3 (4.6) [5.2]	0

( ) = standard deviation [ ] = coefficient of variation, %

* 0% if C_0 (not inoculated) = 5.7 rather than 7.7?

Table B.4a Removal of six surfactants in the OECD Die-Away test – after 8 days

Laboratory Number 4

Surfactant	n	Concentration (mg l^{-1})					% Removal in 8 days		
		Batch 1			Batch 2		Flask		
		Day 0	Day 8		Day 0	Day 8	1	2	3
			Flask 1	Flask 2	C_0	Flask 3			
Soft	1	3.67	4.23	4.03	-	-	0	0	
Anionic	2	3.83	-	4.69				0	
	3	4.44	-	-					
	mean						0	0	
Hard	1	4.25	4.71	4.63			0	0	-
Anionic	2	3.89	5.13	4.51			0	0	-
	3	3.52	-	-					-
	mean						0	0	-
Cationic A	1	4.55	4.50	-			0	-	-
	2	4.65	5.11	-			0	-	-
	3	4.47	5.50	-			0	-	-
	mean						0	-	-
Cationic B	1	4.42	0.58	0.27			86.9	93.9	-
	2	4.23	0.28	0.19			93.4	95.5	-
	3	4.41	0.27	0.28			93.9	93.7	-
	mean						91.4	94.4	
							(3.9)	(1.0)	
							[4.3]	[1.0]	
Amphoteric	1	4.84	0.24	0.29	4.63	0.57	95.0	95.0	87.7
A	2	4.75	0.32	0.38	4.79	0.62	93.3	92.0	87.1
	3	5.12	0.33	0.24	?	0.61	93.8	95.3	
	mean						94.0	94.1	
							(0.9)	(1.7)	
							[0.9]	[1.8]	
Amphoteric	1	4.73	0.32	0.22	4.66	1.09	93.2	95.3	76.6
B	2	4.59	0.31	0.19	4.66	1.18	93.2	95.6	74.7
	3	4.53	0.17	0.38	4.87	1.15	96.2	91.6	76.4
	mean						94.2	94.3	75.9
							(1.7)	(2.3)	(1.0)
							[1.8]	[2.4]	[1.3]

( ) = standard deviation [ ] = coefficient of variation, %

Table B.4b Removal of six surfactants in the OECD Die-Away test – after 14 days

Laboratory Number 4

Surfactant	**n**	**Concentration (mg l^{-1})** Batch 1			**% Removal in 14 days**	
		Day 0	Day 14			
		C_0	Flask 1	Flask 2	Flask 1	Flask 2
Soft Anionic	1	3.67	4.19	0.01	0	99.7
	2	3.83	3.54	0.01	7.6	99.7
	3	3.44	3.75	-0.01	15.6	100
	mean				7.7*	99.8
					(7.8) [101]	(0.2) [0.2]
Hard Anionic	1	4.25	1.36	2.35	68.0	44.7
	2	3.89	1.68	2.53	56.8	35.0
	3	3.52	1.74	2.11	50.6	40.1
	mean				58.5	39.9
					(8.8) [15]	(4.9) [12]

( ) = standard deviation [ ] = coefficient of variation, %
* decided to omit as a flyer

Table B.4c Removal of six surfactants in the OECD Die-Away test – after 19 days

Laboratory Number 4

Surfactant	n	Concentration (mg l^{-1})					% Removal in 19 days		
		Batch 1			Batch 2		Flask		
		Day 0	Day 19		Day 0 C_0	Day 19 Flask 3	1	2	3
Soft Anionic	1	3.67	3.98	0.01			0	99.7	-
	2	3.83	3.81	0.05			0.5	98.7	-
	3	3.44	4.10	0.05			0	98.9	-
	mean						0.2*	99.1	
							(0.3)	(0.5)	
							[150]	[0.5]	
Hard Anionic	1	4.25	0.92	1.27			78.4	70.1	-
	2	3.89	1.20	1.07			69.2	72.5	-
	3	3.52	1.29	1.22			63.4	63.5	-
	mean						70.3	69.3	-
							(7.5)	(3.7)	
							[10.7]	[5.3]	
Cationic A	1	4.55	3.31	-			27.3	-	-
	2	4.65	2.74	-			41.1	-	-
	3	4.47	3.84	-			14.1	-	-
	mean						27.5	-	-
							(13.5)		
							[49]		
Cationic B	1	4.42	0.25	0.21			94.3	95.2	-
	2	4.23	0.19	0.18			95.5	95.7	-
	3	4.41	0.25	0.20			94.3	95.5	-
	mean						94.7	95.5	
							(6.9)	(0.3)	
							[7.3]	[0.3]	
Amphoteric A	1	4.84	-0.02	-0.02	4.63	-0.01	100	100	100
	2	4.75	-0.01	-0.01	4.79	-0.01	100	100	100
	3	5.12	-0.01	-0.01	-	-0.003	100	100	100
	mean						100	100	100
Amphoteric B	1	4.73	0.04	0.18	4.66	0.07	99.2	96.2	98.5
	2	4.59	0.06	0.07	4.87	0.01	98.7	98.5	99.8
	3	4.53	0.07	0.12	-	0.02	98.5	97.5	–
	mean						98.8	97.4	99.1
							(0.4)	(1.2)	(-)
							[0.4]	[1.2]	[-]

( ) = standard deviation [ ] = coefficient of variation, %
* Decided to omit as a flyer

Table B.4d "Abiotic" removal of six surfactants in the OECD Die-Away test – after 8 & 19 days

Laboratory Number 4

Surfactant	n	Concentration (mg l^{-1})			% Removal at	
		Day 0 (C_o)	Day 8 (C_{8d})	Day 19 (C_{19d})	Day 8	Day 19
Cationic A	1	3.98	2.72	1.24	31.7	68.6
	2	4.33	2.72	1.23	37.2	71.6
	3	-	-	-	-	-
	mean				34.5	70.2
Cationic B	1	4.93	4.18	4.93	15.2	0
	2	4.78	4.42	4.93	7.5	0
	3	-	-	-	-	-
	mean				11.4	0
Amphoteric A	1	4.82	5.22	5.17	0	0
	2	4.82	5.19	4.99	0	0
	3	-	-	-	-	-
	mean				0	0
Amphoteric B	1	5.06	4.19	4.91	17.2	3.0
	2	4.67	4.14	4.48	11.3	4.1
	3	-	4.35	4.79	-	-
	mean				14.2	3.5

Table B.5 Removal of 4 surfactants in the OECD Die-Away test – (Total & Abiotic)

Laboratory Number 5

Surfactant	n	Concentration (mg l^{-1})						% Removal			
		Inoculated			Not inoculated			Total		Abiotic	
		C_o	C_{8d}	C_{14d}	C_o	C_{8d}	C_{14d}	8 d	14/19d	8 d	14/19d
Soft	1	4.7	4.7	0.2	4.6	4.8	4.7	0	95.7	0	0
Anionic	2	-	4.7	0.2	-	4.7	4.7	0	95.7	0	0
	3	-	4.7	0.2	-	4.6	4.7	0	95.7	0	0
	mean							0	95.7 (0) [0]	0	0
Hard	1	4.8	4.9	4.9	5.0	4.9	5.0	0	2	0	0
anionic	2	-	4.9	4.7	-	5.0	5.0	0	0	2	0
	3	-	4.9	5.0	-	4.9	5.0	0	2	0	0
	mean							0	1.3 (0.9) [70]	0.7 (1) [157]	0
Cationic B	1	4.4	2.2	1.1	3.9	2.0	1.2	50.0	75.0	48.7	69.2
	2	-	2.7	1.1	-	2.2	1.2	38.6	75.0	43.6	69.2
	3	-	1.9	1.7	-	1.6	1.1	56.8	61.4	59.0	71.8
	mean							48.5 (9.2) [19]	70.4 (7.8) [11]	50.5 (7.8) [18]	70.1 (1.5) [2.2]
Amphoteric	1	5.2	0.3	0.1	5.0	0.1	0.1	94.2	98.1	98.0	98.0
A	2	-	0.1	0.1	-	0.1	0.1	98.1	98.1	98.0	98.0
	3	-	0.1	0.1	-	0.1	0.1	98.1	98.1	98.0	98.0
	mean							96.8 (2.3) [2.4]	98.1 (0) [0]	98.0 (0) [0]	98.0 (0) [0]

( ) = standard deviation [ ] = coefficient of variation, %

Table B.6a Removal of five surfactants in the OECD Die-Away test after 8 & 19 days

Laboratory Number 6

Surfactant	n	Concentration (mg l^{-1})				% Removal of surfactant		
		C_0	C_{8d}	C_{14d}	C_{19d}	8 day	14 day	19 day
Soft	1	0.390*	0.105	0.102	-	73.1	73.9	-
Anionic	2	0.314	0.102	0.101	-	67.4	67.8	-
	3	0.318	0.100	0.100	-	69.6	68.6	-
	mean					70.0	70.1	-
						(3.0)	(3.2)	
						[4.2]	[4.6]	
Hard	1	0.520*	0.475	0.473	-	8.7	9.0	-
Anionic	2	0.460	0.426	0.417	-	0	2.1	-
	3	0.386	0.356	0.320	-	7.8	17.1	-
	mean					5.5	9.4	-
						(2.5)	(7)	
						[42]	[77]	
Cationic B	1	5.7	0.35	-	0.40	93.9	-	93.0
	2	6.6	0.35	-	0.32	94.7	-	95.2
	3	5.8	0.40	-	0.28	93.1	-	95.2
	mean					93.9	-	94.5
						(1)		(1.2)
						[1]		[1.3]
Amphoteric	1	4.1	0.65	-	0.002	84.2	-	100
A	2	5.0	0.85	-	0.002	83.0	-	100
	3	5.8	0.75	-	0.001	97.1	-	100
	mean					84.8	-	100
						(2.1)		(-)
						[2.5]		[-]
Amphoteric	1	4.8	0.75	-	0.006	84.4	-	100
B	2	5.6	0.85	-	0.012	84.8	-	99.8
	3	6.8	0.85	-	0.014	85.8	-	99.8
	mean					85.0	-	99.9
						(1)		(0.1)
						[1.2]		[0.1]

( ) = standard deviation [ ] = coefficient of variation, %
* absorbances, not mg l-1

Table B.6b "Abiotic" removal of five surfactants in the OECD Die-Away test

Laboratory Number 6

Surfactant	Concentration*			% Removal at	
	Day 0	Day 8	Day 20	Day 8	Day 20
Soft anionic	0.136	0.133	0.133	2.2	2.2
Hard Anionic	0.292	0.284	0.278	2.7	4.8
Cationic B	1.367	1.357	1.348	0.7	1.4
Amphoteric A	0.961	0.956	0.948	0.5	1.4
Amphoteric B	0.484	0.486	0.479	0	1.0

* expressed as absorbances